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Rigidity of the Subunit Interfaces of the Trimeric Glutamate Transporter GltT During Translocation

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Glutamate transporters are trimeric membrane proteins in which each protomer contains a separate translocation path. To determine whether structural rearrangements take place at the subunit interfaces during transport, intersubunit disulfide bridges were introduced in the bacterial transporter GltT. None of the intersubunit cross-links, which had been designed across the entire interface, affected the glutamate transport activity, indicating that the subunit interfaces are rigid during turnover.

Keywords: membrane protein; glutamate transport; oligomeric state; transport mechanism; subunit interaction

Background

Glutamate transporters form a large group of membrane proteins that catalyze the uptake of glutamate into cells. The thermodynamically unfavorable concentrative glutamate uptake is driven by coupled transport of protons, sodium ions and/or potassium ions down their electrochemical gradients across the membrane. In mammalians the transporters are involved in clearance of the neurotransmitter glutamate from the synaptic cleft, and in prokaryotes the transporters are involved in the uptake of nutrients, such as glutamate and aspartate.[1] The crystal structure of the aspartate transporter Glt_{ph} from the archaeon *Pyrococcus horikoshii* has been solved.[2] The protein is a homotrimer, and each protomer contains a substrate translocation path. The trimeric oligomeric state is conserved in bacterial and mammalian glutamate transporters.[2–6] Much progress has been made in the identification of the structural determinants of the substrate and cation binding sites of Glt_{ph} and mammalian transporters by crystallographic and mutagenesis experiments.[7–9] But why these proteins are trimeric remains unclear. Here, we show by immobilizing the subunit interfaces in the bacterial glutamate transporter GltT that large structural rearrangements at the interfaces are not required for substrate translocation.

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Cross-linking of the interfaces

In the crystal structure of the aspartate transporter Glt_{ph} each of the interfaces between the protomers consists of three contact regions (Figure 1): Transmembrane helix 2 of one protomer and helix 4a of its neighbor; helices 4b of two neighboring protomers; and helix 5 of one protomer and helices 4c and 5 of its neighbor. Pairs of residues (one on either side of the interface) were selected with the C^{β} atoms 4–6 Å apart in the structure of Glt_{ph}. The amino acids at the homologous positions in the glutamate transporter GltT from *Bacillus stearothermophilus* were mutated to cysteines to allow intersubunit disulfide bond formation. GltT was chosen for these experiments because its activity can be measured readily. GltT shares 36% identical residues with Glt_{ph} and is also trimeric.[6] Double cysteine residues were introduced in all three regions forming the interface: between helix 2 and 4a (I40C/V135C and K46C/K140C), helix 4b and 4b (F143C/T147C and G144C/T147C), helix 4c and 5 (G164C/A185C and G164C/Y188C) and between neighboring helices 5 (K171C/Q177C, K171C/Q180C, P174C/Q177C and V175C/F178C).

The double cysteine mutants and a cysteine-less control were expressed in *Escherichia coli*, as described.[10,11] Membrane vesicles with a right-side-out orientation were isolated and glutamate transport activity was measured, as described in the legend to Figure 2. These experiments were done under reducing conditions (10 mM DTT) in order to prevent disulfide bond formation and thus to be able to compare the activities of the double mutants with the cysteine-less mutant. The cysteine-less mutant

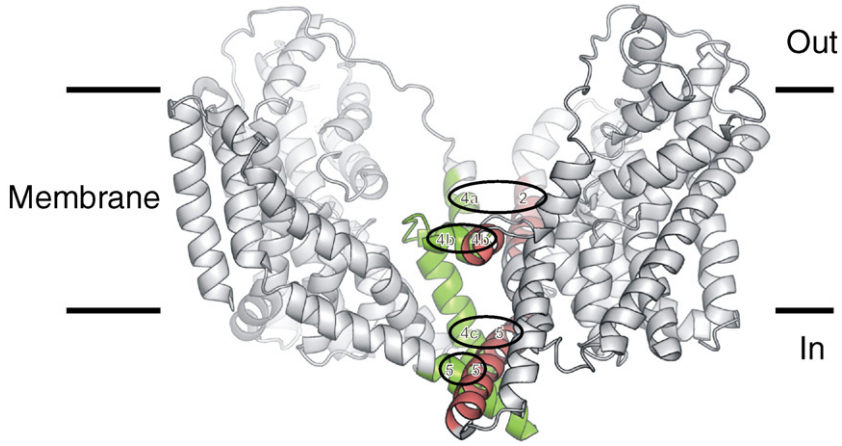

Figure 1. Structural model of Glt_{ph} (PBD accession code 1XFH) seen from the membrane plane. One of the three protomers was deleted to allow a clearer view of the interface between the two remaining protomers. The contact regions are colored red and green in either protomer. Numbering of the helices as by Yernool *et al.*[2] The structure was viewed in PyMOL [http://www.pymol.org].

displayed an initial uptake rate of 10.8 pmol s^{-1} mg^{-1} of membrane protein. Most double cysteine mutants had lower activities (Figure 2(a)), but the differences correlated well with the reduced expression levels of the mutants as estimated from Western blots (Figure 2(b)), indicating that the specific activities of the mutants were similar. When the expression levels were taken into account (as quantified from the Western blots), only the mutants I40C/V135C and K46C/K140C appeared to have lower specific activities than the cysteine-less mutant, but the differences were less than ~twofold. It must be noted though that quantification of the expression levels from the Western blots may not be very accurate, because the transfer of GltT from SDS–PAGE gels to the blot membranes was somewhat variable.

To determine whether the double cysteine mutants could form intersubunit disulfide bridges across the interfaces of the protomers, membrane vesicles were treated with either the oxidizing reagent copper phenantroline or the reducing agent DTT, to catalyze or prevent disulfide bond formation, respectively. Vesicles were run on SDS–PAGE gels, followed by Western blotting and detection of the His_6-tags, which were present at the N termini of all the mutants. Eight mutants, I40C/V135C, K46C/K140C, F143C/T147C, G144C/T147C, G164C/Y188C, K171C/Q177C K171C/Q180C and P174C/Q177C, were cross-linked readily by copper phenantroline and showed bands on the Western blots of the molecular mass of GltT trimers (Figure 3(a)). It is important to note that cross-linking was very efficient and that these mutants were completely converted into covalently cross-linked trimers with no residual monomers or dimers remaining. This shows that both cysteine residues in the double cysteine mutants must be involved in the cross-link formation. If one cysteine only had been involved in the cross-linking, monomers and dimers would be expected in non-reducing SDS–PAGE. For all mutants the optimal cross-linking conditions (copper phenantroline concentration, incubation time and temperature) were determined (shown in Figure 3(b) for I40C/V135C). Two mutants (V175C/F178C and G164C/A185C) could not be cross-linked under any of the conditions tested and these were taken along in the further analysis as negative controls. Two other mutants (K46C/K140C and G164C/Y188C) formed very stable cross-linked trimers that could be reduced only under conditions that are incompatible with activity assays (50 mM β-mercaptoethanol in denaturating loading buffer for SDS–PAGE gels). These mutants were excluded from the experiments described below.

To determine whether cross-linking of the protomer interfaces in the GltT trimer affected the transport activity, glutamate uptake was measured in membrane vesicles that had been treated either with copper phenantroline or with DTT. Oxidation with copper phenantroline was not expected to affect the activities of the cysteine-less mutant and the two double mutants that were used as negative controls (V175C/F178C and G164C/A185C). However, their initial transport rates were reduced by ~25% in the presence of the reagent (Figure 4). It is known that Cu^{2+} binds tightly to *E. coli* membrane vesicles,[12] and that the oxidizing conditions may affect their integrity, leading to slightly leaky vesicles. The shape of the uptake traces of the negative controls are consistent with this notion (Figure 4). Washing of the membrane vesicles with EDTA to remove excess copper ions partially reversed the effect, and treatment with DTT fully restored activity (not shown), but the latter was not compatible with maintaining disulfide cross-links in the double cysteine mutants. Therefore, we used the activity of the cysteine-less mutant as benchmark to determine the effect of intersubunit cross-linking on the transport activity of the mutants. The initial transport rates of all double cysteine mutants were affected to a similar extent by copper phenantroline treatment as the cysteine-less mutant (Figure 4). The ratios between the initial uptake rates in the oxidized and reduced vesicles were calculated for each mutant. *T*-tests showed that there were no significant differences between the ratio of any of the double cysteine mutants and that of the cysteine-less mutant.

Conclusions

Our work shows that it is very likely that the protomer interfaces of the glutamate transporter GltT

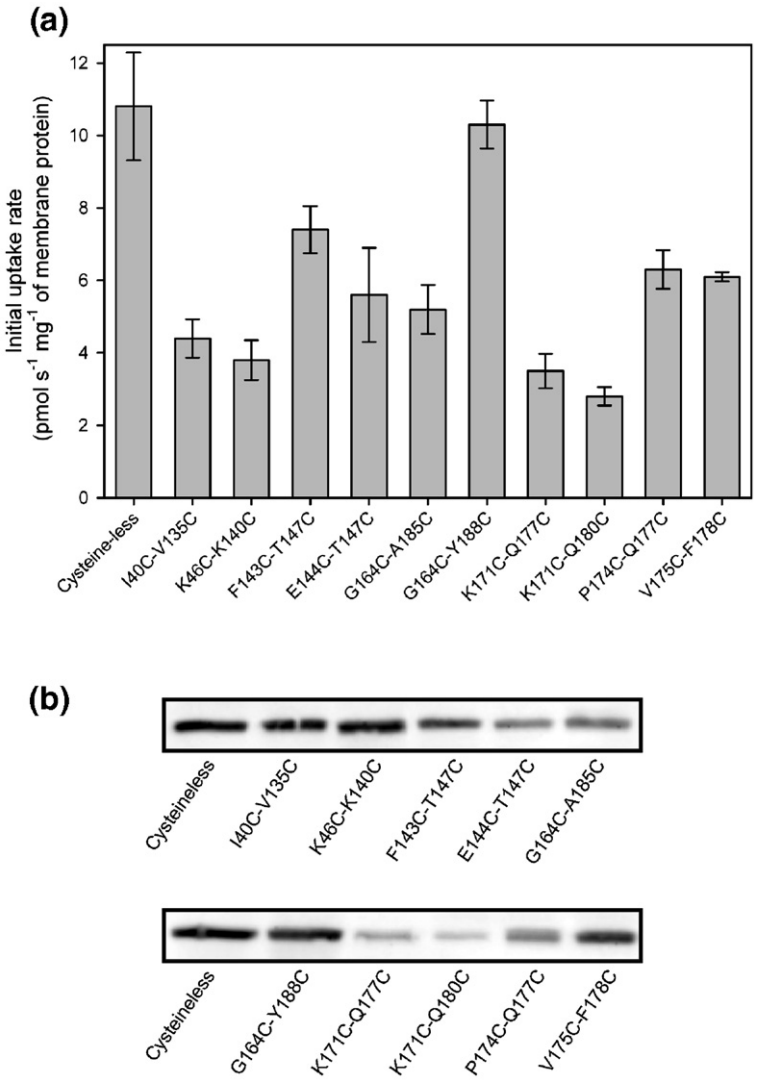

Figure 2. Glutamate transport activities and expression levels of the mutants. (a) The GltT mutants were expressed in *E.coli* ECOMUT2 cells that lack the gene for the endogenous glutamate transporter GltP[10]. Transport was assayed in membrane vesicles with a right-side-out orientation[22] under reducing conditions (10 mM DTT throughout). The vesicles were concentrated to a final protein concentration of 15 mg/ml in 100 mM potassium acetate, 10 mM Mops/*N*-methyl-D-glucamine (pH 7.0). Transport was initiated by diluting 4 μl of vesicle suspension into 400 μl of 110 mM Mops/methylglucamine, 1 μM valinomycin, 0.395 μM [^{14}C]glutamic acid (pH 7.0) thereby creating a membrane potential (diffusion of potassium ions mediated by valinomycin) and a proton gradient (diffusion of acetic acid through the membrane).[23] Transport of glutamate was measured at 20 °C under constant stirring. The uptake was stopped at various time points by addition of 4 ml of 0.1 M ice-cold lithium chloride, followed by rapid filtration over 0.2 μm pore size nitrocellulose filters. Filters were washed once with lithium chloride and then dissolved in 2 ml of scintillation liquid and radioactivity was counted. Initial uptake rates were calculated from the linear part of the uptake curves, which was within the first 10 to 20 s (cf. Figure 4). The protein concentrations of the membrane vesicles were determined by the BCA protein assay kit (Pierce) with bovine serum albumin as standard. The values are the averages of the initial uptake rates from six measurements using three independent vesicle preparations; standard deviations are indicated. (b) Expression levels of the GltT mutants. Membrane vesicles (30 μg of protein) were run on SDS–PAGE gels under reducing conditions and proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Western blots were probed with anti-Histag antibodies that recognized the His_6-tags present on the N termini of all the mutants.

are rigid during catalysis, because none of the inter-subunit cross-links that were introduced across the interfaces affected the glutamate transport activity (Figure 4). Therefore, it is very difficult to envisage a model in which structural rearrangements between the protomers in the glutamate transporter trimers would be required for translocation. This is in agreement with functional studies on the rat and human glutamate transporters EAAC1, EAAT3 and EAAT4 that showed no indication for cooperativity between the protomers in the glutamate binding and transport reactions, nor in the associated anion channel activity.[13–16] For cooperativity to occur it is necessary that information is transmitted from one protomer to another, and consequently that structural rearrangements take place at the protomer interfaces.[17] Similar experiments have shown that structural rearrangements do take place at the interfaces in other proteins, such as the ribosome and an ionotropic glutamate receptor.[18,19]

The trimeric organization is well conserved among archaeal, bacterial and mammalian glutamate transporters.[2–6] Our work shows also that the structural determinants of the interfaces are well conserved between GltT and Glt_{ph}, which share 36% of identical residues. The question thus remains why these proteins are so ubiquitously trimeric. One possibility is that functional interactions other than classical, enzyme-like cooperativity, take place. Recently, it was shown that the trimeric multi-drug transporter AcrB likely acts by a rotary mechanism in which the three protomers are never in the same state and move from one state to another in a coordinated way.[20,21] Rotary mechanisms fit well with the cyclic point group symmetry of oligomeric

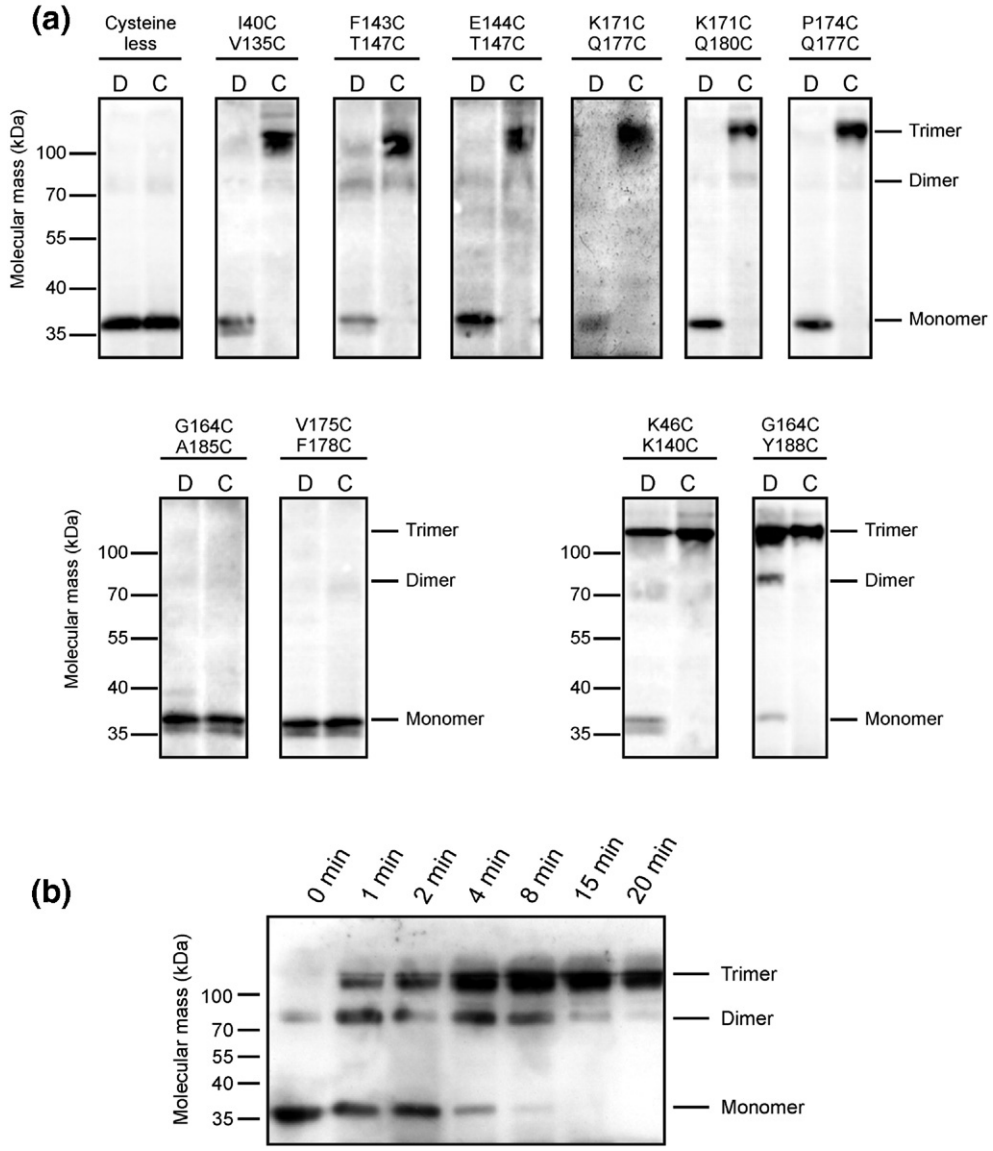

Figure 3. Intersubunit disulfide bond formation. (a) Western blot analysis of membrane vesicles treated with copper phenantroline (lanes labeled C) or dithiothreitol (DTT, lanes labeled D). Copper phenantroline was prepared freshly by mixing in a 1:3 ratio an aqueous solutions of 100 mM $CuSO_4$ and a solution of 100 mM 1,10-phenantroline in dimethylformamide. The final concentration during the reaction was 0.3 mM. To reduce cysteine residues, DTT (10 mM final concentration) was added. Vesicles were incubated at 30 °C for 20 min. The reaction was stopped by addition of 20 mM Na-EDTA, 10 mM *N*-ethyl maleimide (pH 7.0), followed by a 5 min incubation on ice. Vesicles (30 μg of protein) were loaded on SDS–15% PAGE gels, using non-reducing loading buffer. Proteins were transferred from the gels to polyvinylidene difluoride (PVDF) membranes and GltT mutants were detected with antibodies directed against the His_6-tag. The positions of monomeric, dimeric and trimeric GltT are indicated. The apparent double bands observed in some lanes are seen often when the protein runs on SDS–PAGE gels with low acrylamide percentages and they are probably due to incomplete denaturation of GltT. (b) Kinetics of cross-linking of the double mutant I40C-V135C. Membrane vesicles from cells expressing I40C-V135C were treated with copper phenantroline at 30 °C, and the reaction was stopped at the indicated times followed by SDS–PAGE and Western blot analysis with antibodies against the His_6-tag.

membrane proteins. Crystallographic analysis of Glt_{ph} showed that the substrate binding sites in the three protomers in the crystal may not be in identical conformations, which could be indicative of a rotary mechanism, although alternative explanations such as lattice contacts may also explain the differences.[8] The work presented here shows that a rotary mechanism is not likely for the bacterial glutamate transporter GltT because the protomers would have to communicate *via* their interfaces.

Another possible explanation for the trimeric organization of glutamate transporters may lie in a remarkable structural feature of Glt_{ph}: the three protomers surround a single bowl-shaped aqueous basin that reaches half way down the membrane from the outside. It has been suggested that this

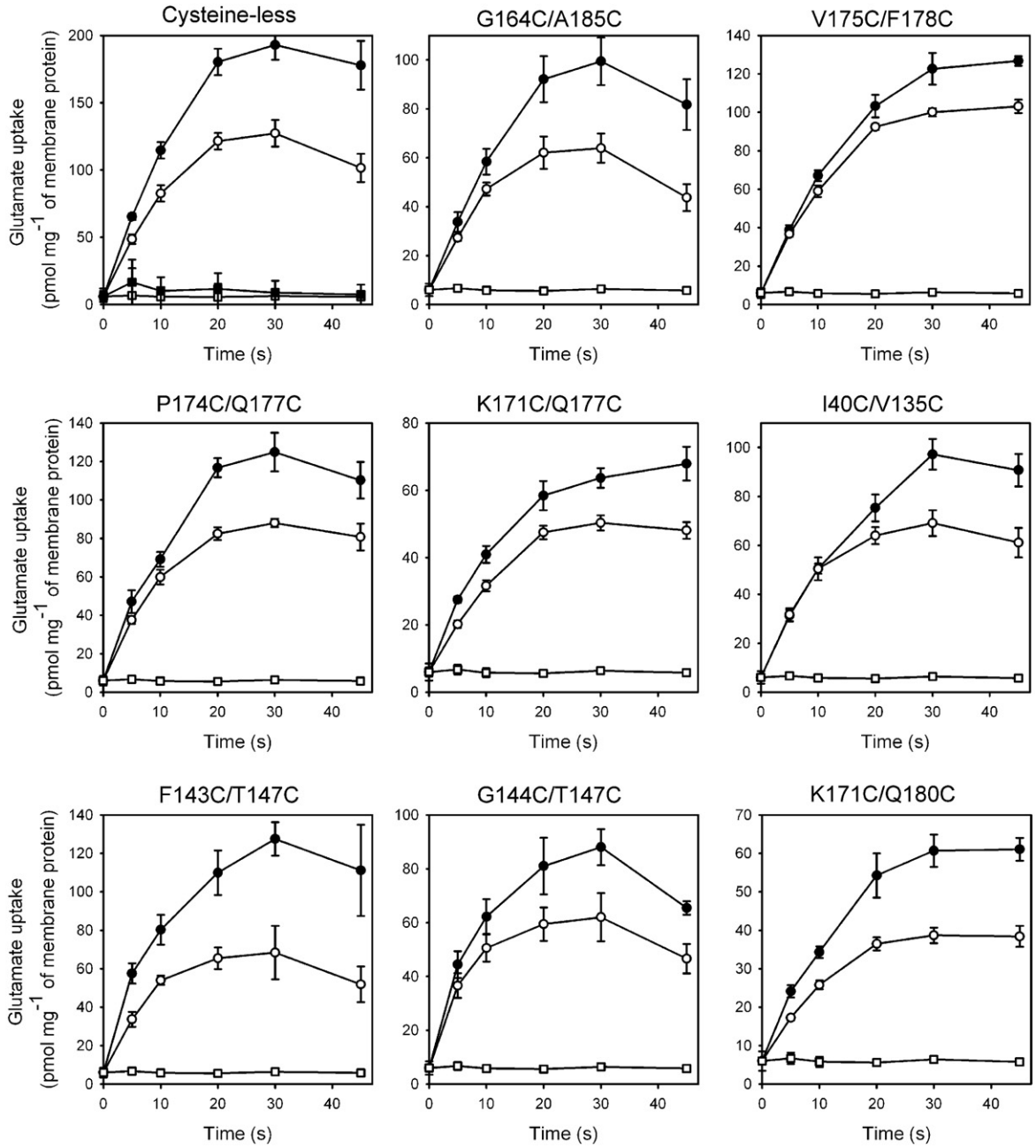

Figure 4. Effects of disulfide bond formation on the glutamate transport rates. Membrane vesicles with a right-side-out orientation expressing the GltT mutants were treated with either copper phenantroline (open circles) or DTT (filled circles) as described in the legend to Figure 3. Glutamate uptake was assayed as described in the legend to Figure 2. As controls, glutamate uptake was measured in the absence of a proton motive force by diluting the vesicles in the 100 mM potassium acetate, 10 mM Mops/*N*-methyl-D-glucamine, 1 μM valinomycin, 0.395 μM [^{14}C]glutamic acid (pH 7.0) (filled squares, trace shown in the panel of the cysteine-less mutant only), or in membrane vesicles from cells harboring an empty expression vector (open squares, shown in all panels). The traces are the averages of six measurements from three independent vesicle preparations. Error bars indicate standard errors of the mean. The glutamate concentration in the assays was well below the K_m (57 μM)[23] to ensure maximum sensitivity to detect cooperative effects.

arrangement may have catalytic advantages for the transport of charged substrate although the exact basis of such a possible advantage is not clear.[2] The transporters may have evolved towards a trimeric structure solely to be able to use the bowl-shaped basin. On the other hand it is also possible that the interfaces between the protomers are more extensive in other conformations than the one found in the crystals of Glt_{ph}. Crystallography analysis of different conformations of glutamate transporters and functional studies of different, distantly related members of the glutamate transporter family will be required to understand the reasons for the trimeric structure of the glutamate transporters.

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